C-myc mRNA expression in epithelial ovarian carcinomas in relation to estrogen receptor status, metastatic spread, survival time, FIGO stage, and histologic grade and type

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Abstract: Recently, it has been suggested that c-myc expression might correlate with estrogen receptor (ER) status and metastatic spread in ovarian cancer. In this study, expression of c-myc mRNA in 90 epithelial ovarian carcinomas was determined using the S1 nuclease protection assay. Expression of c-myc mRNA was detectable in 27 of 90 tumors. There was no significant association between c-myc mRNA expression and metastatic spread, survival time, FIGO stage, or histologic grade and type. C-myc mRNA was expressed in 45% of ER-positive tumors but only 24% of ER-negative tumors ($p = 0.094$; Fisher's exact test). Similarly, 44% of progesterone receptor (PR)-positive and 23% of PR-negative tumors expressed c-myc mRNA ($p = 0.098$). However, the association between c-myc mRNA expression and ER and PR status was not statistically significant. The ratio of mean expression of c-myc mRNA in patients with FIGO stages III/IV compared with patients with FIGO stages I/II was 2.1:1, an insignificant difference ($p = 0.57$, Wilcoxon rank sum test). In conclusion, c-myc was not significantly associated with the clinical parameters investigated in this study.

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C-myc mRNA Expression in Epithelial Ovarian Carcinomas in Relation to Estrogen Receptor Status, Metastatic Spread, Survival Time, FIGO Stage, and Histologic Grade and Type

B. Tanner, M.D., J. G. Hengstler, M.D., A. Luch, Ph.D., R. Meinert, E. Kreutz, M. Arand, Ph.D., C. Wilkens, M.D., M. Hofmann, M.D., F. Oesch, Ph.D., P. G. Knapstein, M.D., and R. Becker, Ph.D.

Summary: Recently, it has been suggested that c-myc expression might correlate with estrogen receptor (ER) status and metastatic spread in ovarian cancer. In this study, expression of c-myc mRNA in 90 epithelial ovarian carcinomas was determined using the S1 nuclease protection assay. Expression of c-myc mRNA was detectable in 27 of 90 tumors. There was no significant association between c-myc mRNA expression and metastatic spread, survival time, FIGO stage, or histologic grade and type. C-myc mRNA was expressed in 45% of ER-positive tumors but only 24% of ER-negative tumors ($p = 0.094$; Fisher's exact test). Similarly, 44% of progesterone receptor (PR)-positive and 23% of PR-negative tumors expressed c-myc mRNA ($p = 0.098$). However, the association between c-myc mRNA expression and ER and PR status was not statistically significant. The ratio of mean expression of c-myc mRNA in patients with FIGO stages III/IV compared with patients with FIGO stages I/II was 2.1:1, an insignificant difference ($p = 0.57$, Wilcoxon rank sum test). In conclusion, c-myc was not significantly associated with the clinical parameters investigated in this study. **Key Words:** C-myc mRNA—Ovarian cancer—S1 nuclease assay—Estrogen receptor—Progesterone receptor—Metastatic spread.

The myc family of cellular oncogenes, c-myc, N-myc, and L-myc, encode three highly related nuclear phosphoproteins. The role of myc-oncogene amplification and/or overexpression in tumorigenesis is still not clear, but the oncoproteins appear to play a central role in the control of cell proliferation and differentiation (1). Amplification and/or overexpression of c-myc has been described in small cell lung carcinomas, cervical carcinomas (2–4), leukemias (5,6), testicular tumors, breast cancers, and colorectal cancers (7). In breast and cervical carcinomas, amplification of c-myc is an independent prognostic factor and correlates with poor prognosis (2,8).

From the Department of Obstetrics and Gynecology, (B.T., E.K, C.W., M.H., P.G.K.), Institute of Toxicology (J.G.H., M.A., F.O., R.B.), and Institute of Medical Statistics and Documentation (R.M.), University of Mainz, Germany.

Address correspondence and reprint requests to Dr. Berno Tanner, Department of Obstetrics and Gynecology, University of Mainz, Langenbeckstraße 1, 55 131 Mainz, Germany.

Previous studies observed amplification and/or overexpression of c-myc in ovarian carcinomas (9–11), suggesting that c-myc activation is involved in the evolution of malignant phenotypes of tumor cells. Furthermore, carcinomas expressing high amounts of c-myc were more aggressive (12,13). However, Baker et al. (11) did not find a significant relationship between c-myc amplification and response to platin-based chemotherapy. Chien et al. (14) reported that a 7.5-fold increase in c-myc mRNA expression could be induced in an estrogen receptor (ER)-positive (28 fmol receptor protein/mg protein) ovarian cancer cell line by 100 nM estrogen, although almost no increase in c-myc mRNA expression was induced in an ovarian cancer cell line with extremely low expression of ER (1 fmol receptor protein/mg protein). Thus, ER status and c-myc mRNA expression might also be associated in ovarian carcinomas *in vivo*. Recently, Volm et al. (15) observed a higher metastatic spread of ovarian carcinomas with positive immunoreactivity of c-myc products, although this result was not

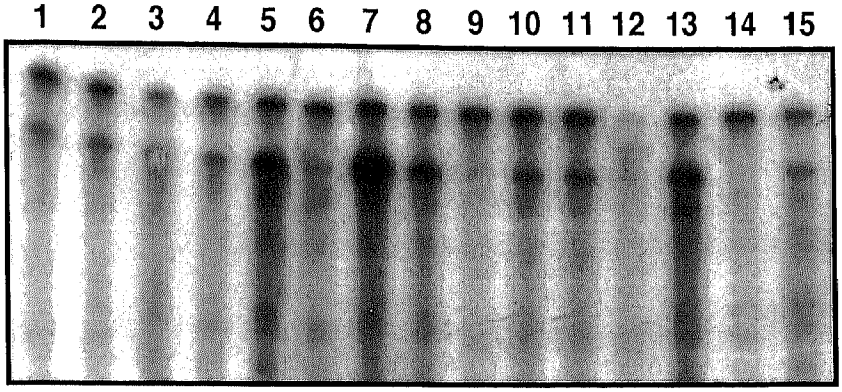

FIG. 1. Representative S1 nuclease protection assay of RNA isolated from ovarian cancer tissue. If c-myc mRNA is expressed in tumor tissue it will hybridize with added 32 p nuclease labelled antisense RNA probe of c-myc and therefore will be protected from digestion by S1 nuclease, which is unable to digest double stranded RNA. The protected fragment results in a specific c-myc signal (2 →), which quantitatively correlates with c-myc mRNA expression. The topmost band (1 →) represents mRNA, which was not completely digested by nuclease S1. For quantification, three standards (lanes 13 to 15) were included in each gel. The intensity of standard 1 (lane 15) was defined as 1 (relative c-myc expression, arbitrary unit), of standard 2 (lane 13) as 10, and of standard 3 (lane 14) as 0. Lanes 1 to 11 represent c-myc mRNA expression of various tumor samples.

statistically significant, possibly due to the relatively small number of patients examined.

In this study, the sensitive S1 nuclease assay was used to quantify expression of c-myc mRNA in tumor tissue of 90 patients with ovarian carcinoma. We examined a possible association between c-myc mRNA expression and survival time, FIGO (International Federation of Gynecology and Obstetrics) stage, and histologic grade and type; a possible correlation between ER and progesterone receptor (PR) status and c-myc mRNA expression; and a possible association between c-myc mRNA expression in primary tumors and metastatic spread.

TABLE 1. *C-myc mRNA expression in tumor specimens from 83 primary ovarian carcinomas and 7 recurrences*[a]

C-myc expression	Frequency (%)
Primary carcinomas (arbitrary units)	
0.0	59 (71.1)
0.3	1 (1.2)
0.4	1 (1.2)
0.5	1 (1.2)
0.6	2 (2.4)
0.7	2 (2.4)
0.8	1 (1.2)
0.9	1 (1.2)
1.2	1 (1.2)
1.3	1 (1.2)
1.5	1 (1.2)
1.7	1 (1.2)
1.8	1 (1.2)
2.0	1 (1.2)
2.4	2 (2.4)
2.5	1 (1.2)
3.5	1 (1.2)
3.7	1 (1.2)
4.2	1 (1.2)
5.5	1 (1.2)
16.6	1 (1.2)
17.2	1 (1.2)
Total	83 (100.0)
Recurrences	
0.0	4 (57.1)
0.4	1 (14.3)
0.5	1 (14.3)
0.7	1 (14.3)
Total	7 (100.0)

[a] C-myc mRNA was determined by the S1 nuclease assay. The extent of c-myc expression was expressed in arbitrary units compared to standards (Fig. 1).

MATERIALS AND METHODS

Tissue Specimen and Pathologic Data

Tumor tissue was collected from 90 patients who underwent surgery from 1986 to 1992 at the Department of Gynecology at the University of Mainz. Tissue specimens were taken intraoperatively, immediately frozen in liquid nitrogen, and stored at −80°C. After 20 cryosections (20 μm thick) were taken, a 7 μm thick cryosection was reviewed by a pathologist (C.W.). Only tissue

TABLE 2. *C-myc mRNA expression in tumor tissue of 83 patients with primary ovarian carcinomas and 7 with recurrences*[a]

	C-myc expression (mean, standard deviation)	No. of cases
Primary carcinomas	0.88, 2.75	83
Recurrences	0.23, 0.29	7
FIGO stage I/II	0.49, 1.23	24
FIGO stage III/IV	1.04, 3.16	59
Grade 1	0.61, 1.21	18
Grade 2/3	0.95, 3.04	65
Serous carcinomas	1.14, 3.40	51
Nonserous carcinomas	0.35, 0.90	26

[a] The expression of c-myc (>0) was not associated with histologic grade, FIGO stage, or histologic type (Wilcoxon rank sum test, $p > 0.05$). The difference between primary carcinomas and recurrences was not significant.

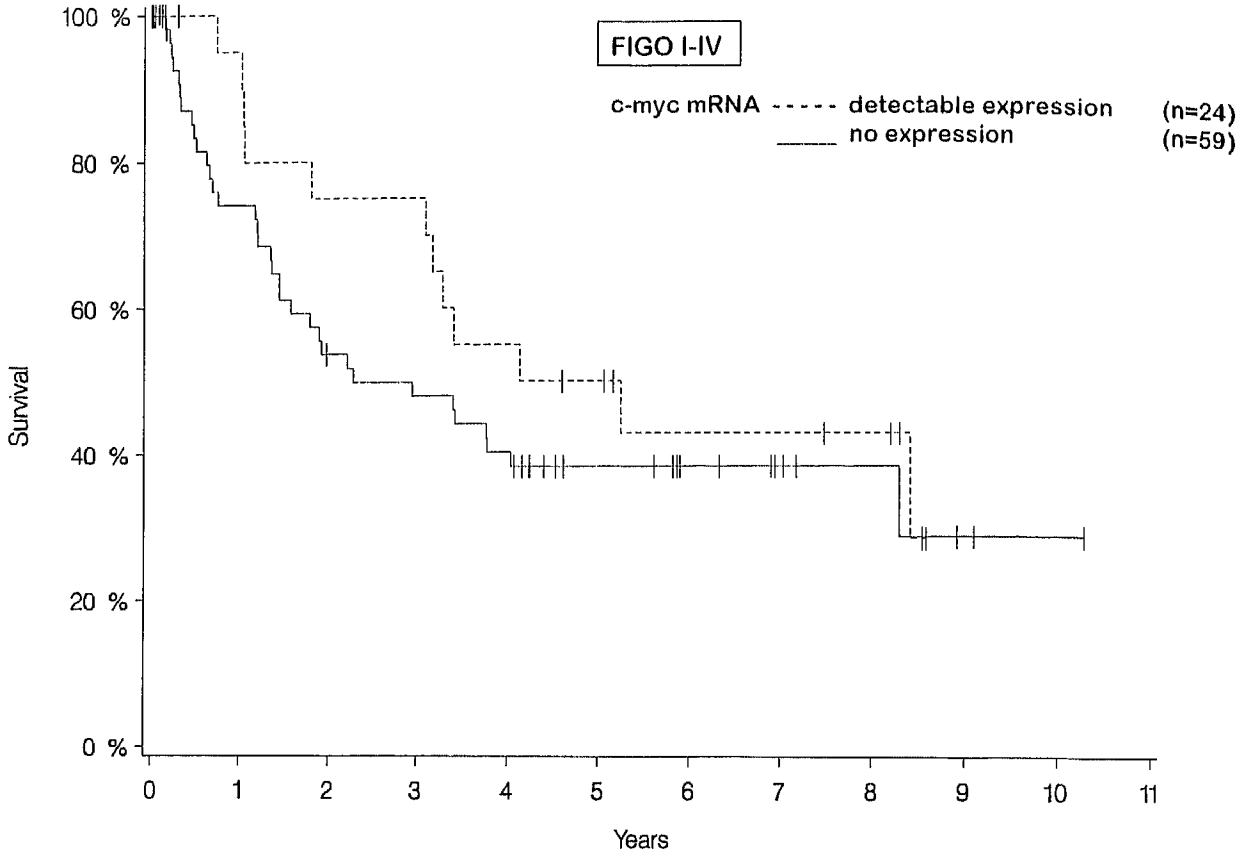

FIG. 2. Survival of 83 patients with primary ovarian cancer (FIGO stages I to IV). The difference between patients with and without detectable expression of c-myc mRNA was not significant ($p = 0.33$; log rank test).

samples with >80% tumor cells in all 7 μm thick cryosections were used for further analysis.

Eighty-three of the 90 patients underwent surgery because of primary cancer and 7 because of recurrent disease. Patients with benign and non-epithelial tumors and borderline tumors were excluded from the study. Treatment of patients with ovarian carcinoma was primarily by total abdominal hysterectomy, bilateral salpingo-oophorectomy, and omentectomy. In patients with stage III and IV disease with recurrences which could not be completely removed, debulking reduced the remaining tumor to a diameter of <2 cm. Postoperative chemotherapy consisted of carboplatin (350 mg/m^2) or cisplatin (50 mg/m^2) and cyclophosphamide (1000 mg/m^2) in 6 cycles.

Histologic typing was performed by WHO criteria (16). Epithelial tumors were subdivided into serous and nonserous carcinomas. Histologic grade of malignancy, which ranged from grade 1 (differentiated) to grade 3 (undifferentiated), was assessed by tumor architecture, amount of solid tumor, nuclear pleomorphism, nuclear-cytoplasmic ratio, number of nucleoli, and mitoses (17). Tumor staging was performed according to FIGO (18). Determination of ER and PR status was performed immunohistochemically as described by Beck et al. (19).

Isolation of RNA and S1 Nuclease Assay

Total RNA was isolated by a single-step method using the guanidinium thiocyanate-phenol-chloroform extraction technique (20). The concentration of RNA was determined spectrophotometrically at 260 nm. After phenol extraction and ethanol precipitation, all RNA samples were stored as aliquots of 20 μg at −80°C until use. All total RNAs prepared from cryopreserved tumor samples were checked for integrity by conventional agarose gel electrophoresis before S1 nuclease assay, by means of the presence of 28S, 18S, and 5S rRNA. If the bands of 28S, 18S, and 5S rRNA were not clearly visible, the respective RNA samples were excluded from the study. In addition, the problem of false negative results as a consequence of mRNA degradation was excluded, as in previous studies (21,22) analyzing the same samples for expression of c-erbB-2 and MDM2 mRNA. The genera-

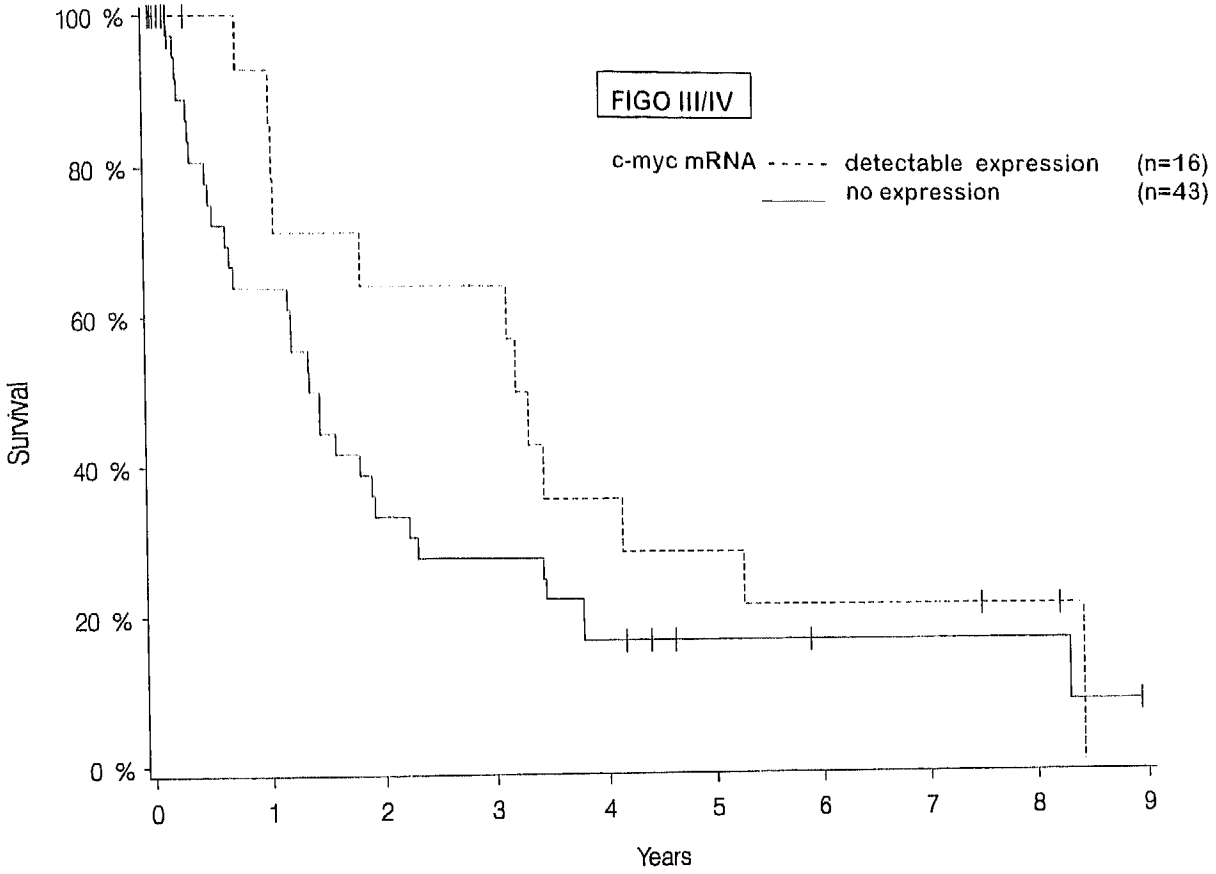

FIG. 3. Survival of 59 patients with primary ovarian carcinomas in FIGO stages III and IV. The difference between patients with and without detectable expression of c-myc mRNA was not significant ($p = 0.21$; log rank test).

tion of the transcripts and the S1 nuclease protection assay were performed following the protocols of Myers et al. (23) and Krieg and Melton (24). The PstI/PstI (430 bp) fragment of the c-myc cDNA (exon 2) was isolated from the plasmid pKH 47 and subcloned into the PstI site of pSP64. To observe an electrophoretic shift, suitable to differentiate between a residual, undigested transcript and a protected message, the Pst/Pst insert of c-myc was artificially elongated by downstream cloning of an irrelevant EcoRI/BamHI fragment from pBR322 into the multiple cloning site of pSP64. Linearization with EcoRI yielded the template for the generation of a ^{32}P labelled antisense transcript using SP6 polymerase (Boehringer, Mannheim). The transcript was further purified by phenol extraction and ethanol precipitation. A total of 10^5 dpm of radioactively labeled transcript (specific activity 10^7 dpm/μg RNA) was mixed with 5 μg of total tissue RNA and 100 μg E. coli tRNA, dried in a speed vac, and resuspended in hybridization buffer (40 mM Pipes, pH 6.4, 1 mM EDTA, 400 mM NaCl, 80% formamide). The RNA mix was denatured by incubation at 90°C for 10 minutes and subsequent hybridization was carried out at 52°C for 3 hours. Fifty units of S1 nuclease (Pharmacia, Uppsala, Sweden) in 300 μg S1-buffer (30 mM sodium acetate, pH 4.6, 50 mM NaCl, 1 mM ZnCl) were added and incubation was continued at 37°C for 60 minutes. After phenol-chloroform extraction and ethanol precipi-

TABLE 3. *Expression of c-myc mRNA in tumor tissue from 83 patients with primary ovarian carcinomas in relation to estrogen and progesterone receptor status*

	c-myc mRNA Expression	
	Detectable (>0)	Not detectable
Estrogen receptor[a]		
% positive (n = 20)	45 (n = 9)	55 (n = 11)
% negative (n = 54)	24 (n= 13)	76 (n = 41)
Progesterone receptor[b]		
% positive (n = 23)	44 (n = 10)	56 (n = 13)
% negative (n = 48)	23 (n = 11)	77 (n = 37)

The differences between c-myc mRNA expression between estrogen receptor-negative and -positive tumors ($p = 0.094$), between progesterone receptor-negative and -positive tumors ($p = 0.098$) were not significant (Fisher's exact test).

[a] Estrogen receptor status was determined in only 74 of 83 patients.

[b] Progesterone receptor status was determined in only 71 of 83 patients.

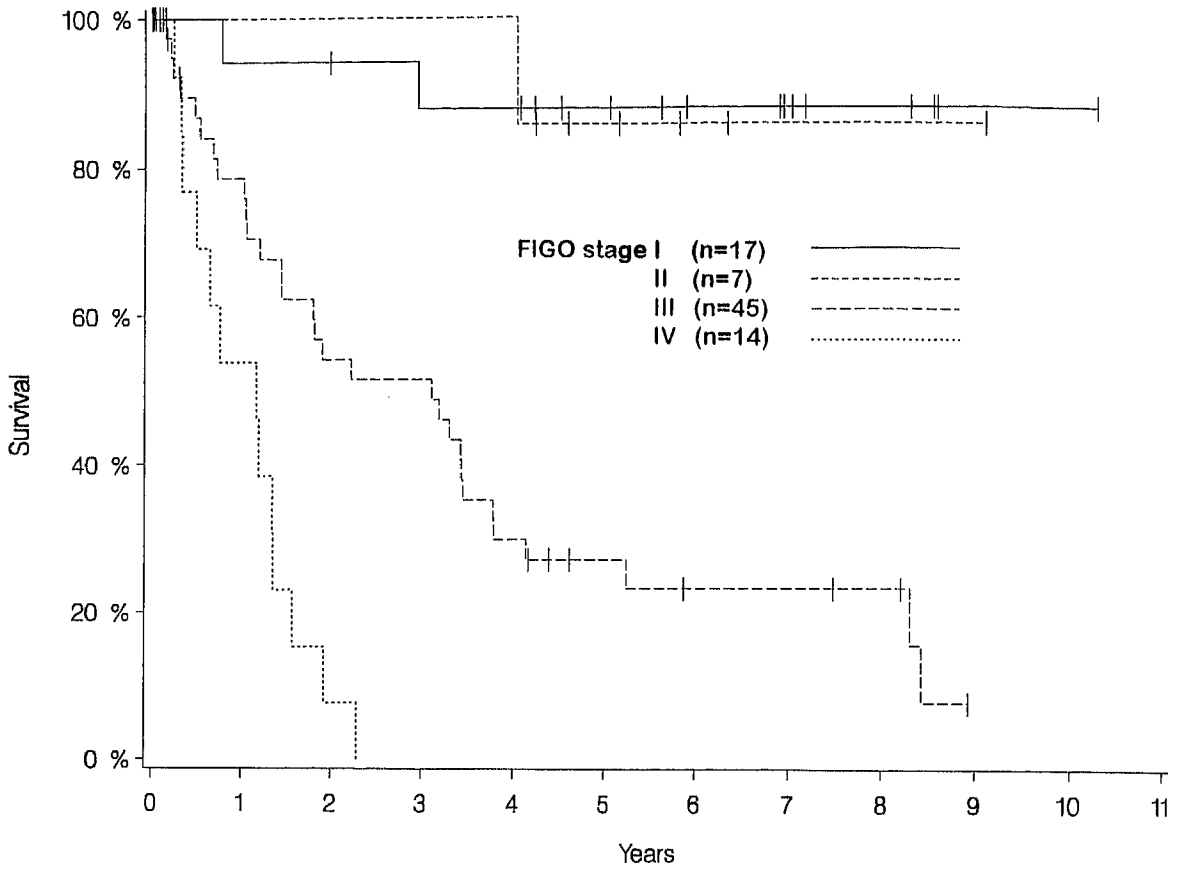

FIG. 4. Survival curves of 83 patients with primary ovarian carcinoma. Survival time correlated significantly with FIGO stage at diagnosis ($p = 0.0001$; log-rank test).

tation, the hybrids were dissolved in loading buffer, heat denatured at 95°C for 15 minutes, and separated on denaturing polyacrylamide gels containing 8 M urea. Detection was performed by autoradiography. Quantitation of RNA expression was carried out by densitometry (densitometer Elscript 400, Kirschmann, Germany). The maximal deviation between two determinations of the same sample was 20%. The mean value of two experiments was calculated. For standardization, mRNA of three ovarian carcinomas were included in each gel (Fig. 1) as standards. The intensity of the band obtained from standard 1 (lane 15) was defined as 1 (relative c-myc expression, arbitrary unit). Compared to standard 1, the intensity of standard 2 (lane 13) was 10 and the intensity of standard 3 (lane 14) was 0 (densitometric determination). A standard curve with standards 1, 2 and 3 was obtained for each gel and used to calculate c-myc expression of each tumor sample. A representative gel is shown in Figure 1.

Statistical Analysis

The association between expression of c-myc mRNA and established prognostic factors, such as histologic grade, FIGO stage, and histologic type, was investigated using the Wilcoxon rank sum test and Fishers' exact test (25). Kaplan-Meier curves were plotted to assess overall survival (25). Different survival curves were compared using the log-rank test. All two-sided p values <0.05 were considerated statistically significant. Statistical analysis was performed using the Statistical Analysis System (SAS, Cary, NC) (26).

RESULTS

Large interindividual differences in the levels of c-myc mRNA expression were observed in different tumor tissue specimens using the S1 nuclease protection assay. Figure 1 shows a representative result with the S1 nuclease assay. The topmost band represents transcripts which were not completely digested by nuclease S1. The subsequent band represents the specific c-myc signal. Based on the signal intensities we defined two categories: no expression of c-myc mRNA (0) and detectable expression (>0).

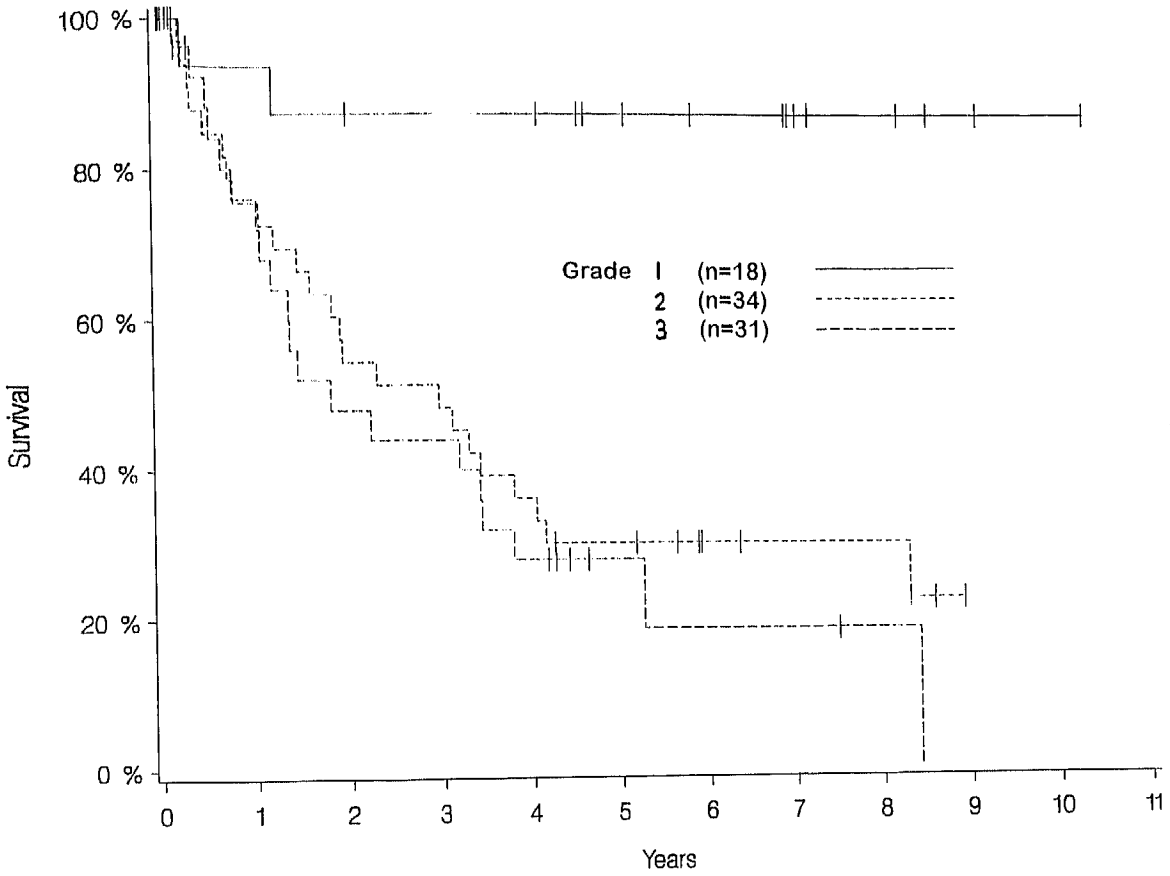

FIG. 5. Survival of 83 patients with primary ovarian carcinoma. Survival time correlated significanctly with histologic grade ($p = 0.0006$; log-rank test).

In 83 primary carcinomas, no c-myc expression was detectable in 59 (71%) of the tumor samples. Twenty-four samples (29%) showed detectable expression. The data for all samples are given in Table 1. In tumors of 7 patients with recurrences, 4 specimens showed no expression of c-myc; 3 showed a detectable expression.

The mean expression of c-myc was 0.88 ± 2.75 (arbitrary units ± standard deviation) in primary carcinomas and 0.23 ± 0.30 in recurrences (Table 2), which did not amount to a significant difference ($p = 0.7$, Wilcoxon rank sum test).

A possible association between c-myc expression and survival time, FIGO stage, and histologic grade and tumor type was examined in 83 primary ovarian carcinomas. The median survival time for patients without c-myc expression was 28 months (Fig. 2), compared with 36 months for those with detectable c-myc expression, an insignificant difference ($p = 0.33$).

The 59 patients with advanced disease (FIGO stages III and IV) were additionally analyzed for a possible association between survival time and c-myc expression. The median survival time for patients without detectable c-myc expression (= 0) was 17 months, compared to 40 months for patients with detectable c-myc expression (Fig. 3), an insignificant difference ($p = 0.21$; log-rank test).

Patients with FIGO stages I and II showed a c-myc expression of 0.49 ± 1.23 (mean ± standard deviation) compared to 1.04 ± 3.16 for patients with FIGO stages III and IV (Table 2), an insignificant difference ($p = 0.57$, Wilcoxon rank sum test). Similarly, no significant association could be shown between c-myc expression and histologic grade. The mean c-myc expression was 0.61 ± 1.21 in grade 1 tumors compared to 0.95 ± 3.04 in grade 2 and 3 tumors (Table 2).

In serous carcinomas, the mean c-myc expression was 1.14 ± 3.40, compared to 0.35 ± 0.90 in tumors of other histologic types (Table 2), an insignificant difference.

In 45% of ER-positive tumors, c-myc mRNA expression was detectable, compared to 24% in ER-negative tumors ($p = 0.094$, Fisher's exact test) (Table 3). Fourty-four percent of the PR-positive and 23% of the PR-negative tumors expressed detectable amounts of c-myc mRNA ($p = 0.098$, Fisher's exact test). Therefore,

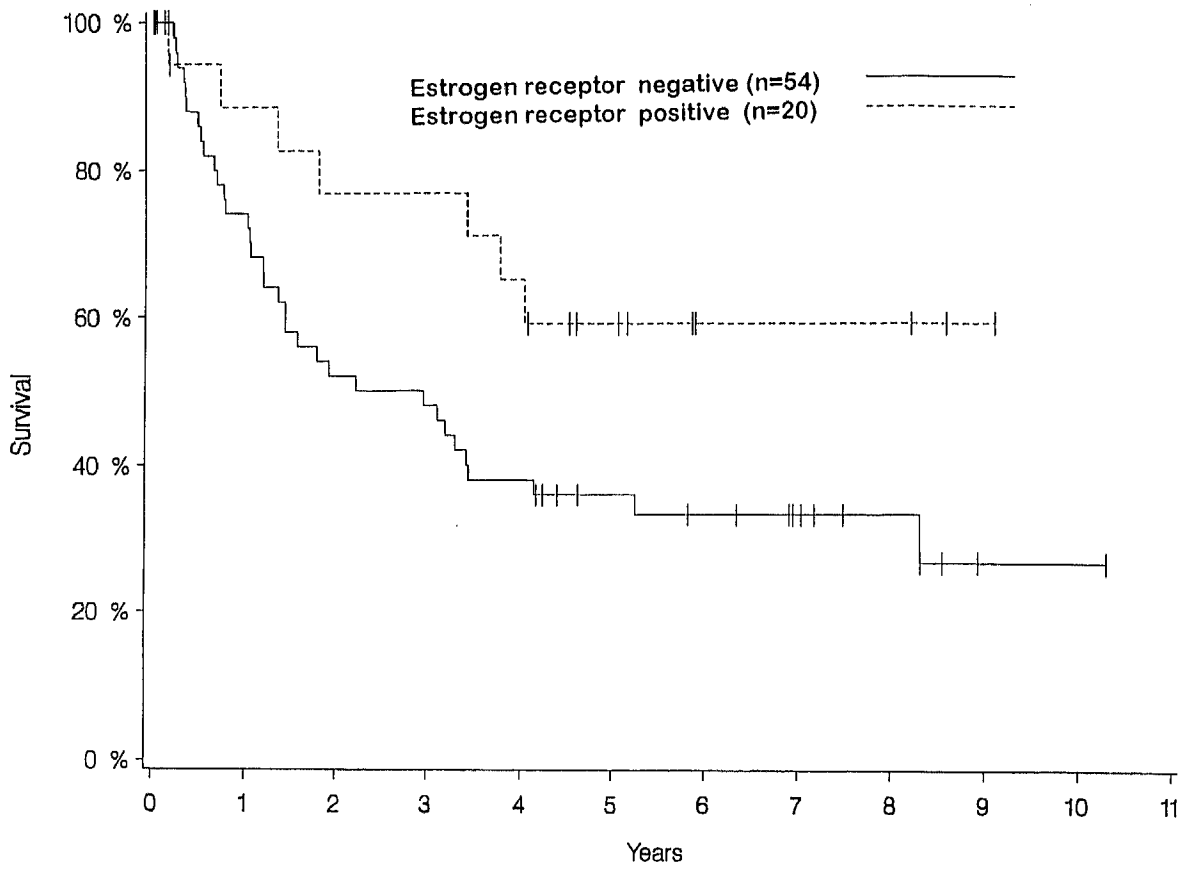

FIG. 6. Survival curves of 74 patients with primary ovarian carcinoma. Survival time correlated significantly with ER status ($p = 0.05$; log-rank test).

expression of c-myc mRNA was higher if ER and PR status was positive. However, the differences did not attain statistical significance.

Mean c-myc mRNA expression in primary tumors of patients with metastatic spread was 0.21 ± 1.32 compared to 0.89 ± 2.15 in primary tumors of patients without metastatic spread (FIGO stages I to III versus FIGO stage IV). Differences between patients with and without metastatic spread were not significant ($p = 0.06$, Wilcoxon rank sum test). Therefore, no significant relationship of c-myc expression with survival time, FIGO stage, histologic grade and type, metastatic spread, and ER and PR status could be shown.

In contrast, FIGO stage and histologic grade at diagnosis were all associated with survival time. At the last follow-up, 21 of 24 patients with Stage I or II ovarian carcinoma were alive, but the median survival time was 37 months for patients with Stage III tumors (Fig. 4) and only 14 months for patients with Stage IV tumors ($p = 0.0001$, log-rank test). Sixteen of 18 patients with histologic grade 1 tumors were alive at the last follow-up, but shorter survival times were observed for patients with grades 2 and 3 (Fig. 5); the median survival time was 35 months for grade 2 tumors and 20 months for grade III tumors ($p = 0.0006$, log-rank test). Furthermore, ER status was associated with survival time. Thirteen of 20 patients with ER-positive tumors were alive at last follow-up, but the median survival time for those with ER-negative tumors was 31 months (Fig. 6, $p = 0.05$, log-rank test). Twenty of 48 patients with PR-positive tumors were alive at last follow-up, and the median survival time of patients with PR-negative tumors was 35 months (Fig. 7, log-rank, $p = 0.1336$).

DISCUSSION

Amplification and/or overexpression of c-myc has been shown to be an independent prognostic factor in breast and cervical cancer (2,8). Gene amplification of c-myc is generally associated with increased expression of its oncoprotein p62 (7), which has been shown to increase before the development of aneuploidy in the evolution of a malignant phenotype (27). Furthermore, c-myc amplification has been reported to correlate with nuclear atypia and high mitotic activity (27).

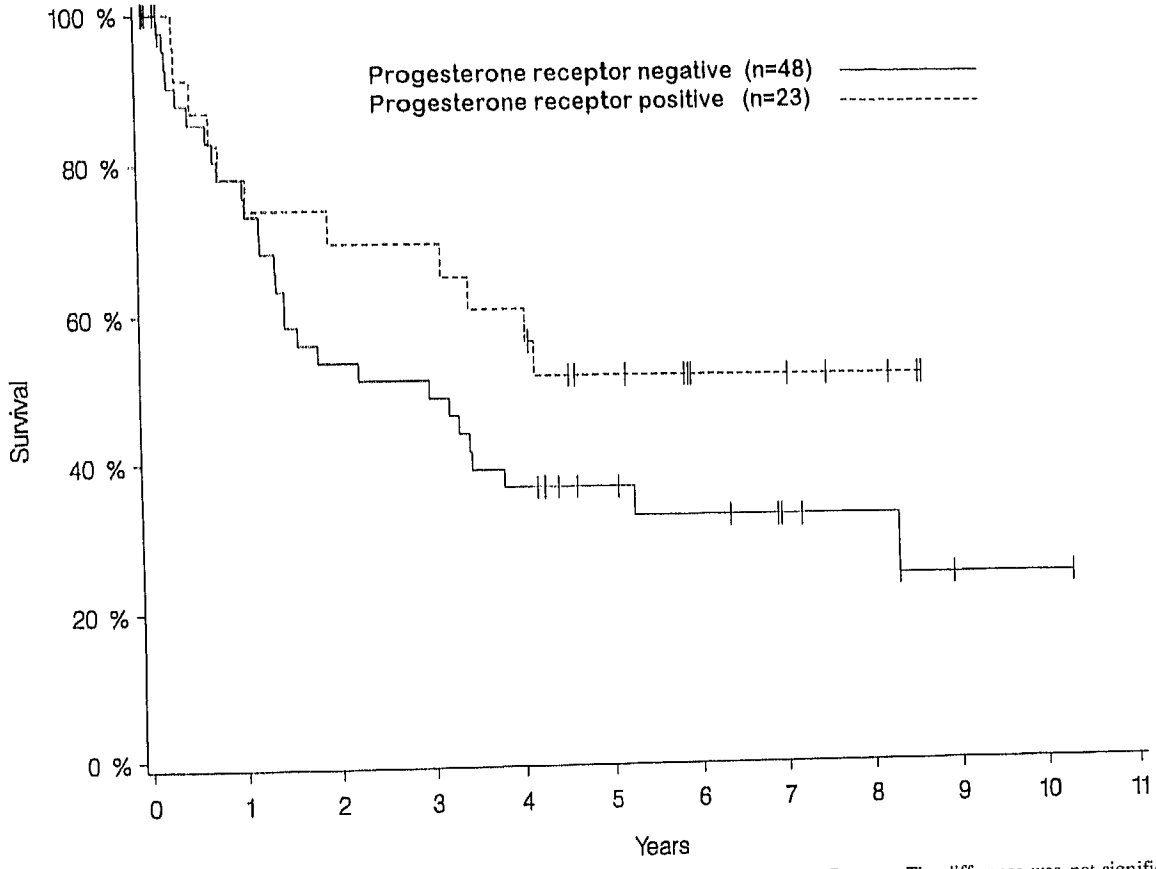

FIG. 7. Survival curves of 71 patients with primary ovarian carcinoma with positive and negative PR status. The difference was not significant ($p = 0.1336$; log-rank test).

The exact role of c-myc in ovarian cancer remains unclear. Bauknecht et al. (12) reported that ovarian carcinomas expressing high amounts of c-myc mRNA were more aggressive, although other investigators did not find an association between c-myc expression and prognosis (10,11,27). Tashiro et al. (9) observed higher expression of c-myc mRNA in FIGO stage III ovarian cancer tissue specimens compared to tissue specimens from normal ovaries. However, FIGO stage IV ovarian cancer specimens did not differ significantly from normal ovaries and FIGO stage I or II ovarian cancer tissue specimens. The increase in FIGO stage III tumors was interpreted as a temporary activation of c-myc mRNA expression during tumor development (9). Recently, Volm et al. (28) reported that metastatic spread might be associated with p62-immunoreactivity in ovarian cancer. Furthermore, evidence was given that estrogen stimulates c-myc mRNA expression in an ER-positive ovarian cancer cell line (14).

In this study, the S1 nuclease protection assay was used to determine c-myc mRNA in a relatively high ($n = 90$) number of ovarian cancer tissue specimens. Recently, we showed that the nuclease S1 protection assay allowed an extremely sensitive and reproducible determination of mRNA expression, which was superior to immunohistochemistry or Northern blot analysis (29). This might be explained by the absence of single-stranded ribonucleic acids, which rehybridize during incubation as do nick translates in the Northern blot. Furthermore, hybridization is performed entirely in the liquid phase (29).

In this study, c-myc mRNA expression was detected in 30% of 90 ovarian carcinomas. As reported in previous studies (30–33), survival time significantly correlated with FIGO stage and histologic grade. No significant association between c-myc mRNA expression and histologic grade and type, survival time, or metastatic spread was observed. Mean expression of c-myc was 2.1-fold higher in patients with FIGO stages III/IV compared to patients with FIGO stages I/II. However, this difference was not significant due to large interindividual variability in c-myc mRNA expression, even in patients with identical FIGO stages.

A higher expression of c-myc mRNA was observed in

ER-positive tumors. Expression of c-myc was detected in 45% of ER-positive tumors, compared to only 24% in ER negative tumors, analogous to the study in cell lines by Chien et al. (14), who observed an association between ER status and c-myc expression in these lines. However, the difference in c-myc mRNA expression between ER-positive and ER-negative tumors was not significant in our study. A tendency towards higher expression of c-myc mRNA was observed in ER- and PR-positive ovarian carcinomas, but none of the clinical parameters investigated in this study were significantly associated with c-myc expression.

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